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Review

# A review and assessment of the potential use of RNA:DNA ratios to assess the condition of entrained fish larvae

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ABSTRACT

In rivers, lakes, and other aquatic systems throughout the world, intake pipes withdraw huge volumes of water for industrial purposes, including power plant cooling. During this process, large numbers of small-bodied, early life-stages of fish are pulled into pipes (i.e., entrained) and may be subjected to physical, thermal and chemical stress. As a result of such entrainment, these organisms can suffer direct or indirect mortality. However, given that the vast majority of larval fish are likely to die during early life due to natural processes, it is not obvious that entrainment-related mortality will have a strong influence on subsequent adult population sizes. The ability to evaluate if larval fish are dead on arrival, moribund, or in poor condition (i.e., likely to die through natural processes) at the time of entrainment could shed light on likely population-level impacts. To this end, we review the potential use of RNA:DNA ratios to index condition of entrained larval fish. Through a meta-analysis of published research studies, we demonstrate that RNA:DNA ratios of larval fish are responsive to starvation stress, with effect size increasing with duration of starvation. We relate these results to a surrogate measure of irreversible long-term negative impacts to fish populations, and demonstrate that the timescale over which RNA:DNA ratios respond to stress may not be long enough to reflect before-and-after entrainment stress. We also highlight the diverse factors contributing to variation of RNA:DNA ratios, including methodological, ontogenetic, and thermal influences. We believe that the need to account for these influences when comparing among RNA:DNA values limits the utility of broadly using RNA:DNA ratios to evaluate entrainment effects. However, the method shows promise as a quick and efficient means of determining fish condition and, used in proper context (e.g., specific to a given set of environmental conditions; in conjunction with other assessment techniques), may provide a powerful tool in assessing the effects of entrainment on fish populations. Assuming that researchers can account for sources of background variation, RNA:DNA analyses may be most useful for assessing the condition of fish larvae susceptible to entrainment (i.e., physically in the vicinity of the water intake) and/or evaluating whether fish larvae are likely to die from natural processes independent of entrainment.



**Contents**



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## 1. Introduction

The early life stages of fish and shellfish are either truly passive (non-motile and drifting) or relatively poor swimmers, essentially at the mercy of physical forces within the water body for transport. As a consequence, those organisms that are transported into the area of influence of a cooling water intake may be drawn in to the intake system (i.e., entrained). Entrainment of larval fishes and eggs is a particular concern for power generation facilities that withdraw large volumes of ambient water from freshwater and marine systems to cool condensers. Fishes that are carried into the power plant with cooling water may be negatively impacted by heat and/or rapid thermal changes (Cada et al., 1982; Wismer and Christie, 1987), turbulence and/or abrasion (Marcy et al., 1978; Cada et al., 1982), chemicals (Bamber and Turnpenny, 2012), or by synergistic effects of multiple stressors. Some studies have suggested that larval fishes can survive entrainment, though discharge temperatures above 32 °C appear to negatively affect survival (Mayhew et al., 2000; EPRI, 2009). Others suggest that, when considering the full suite of impacts that an entrained organism might experience, short-term stresses associated with the entrainment process would lead to almost 100% mortality of larval fishes (Kelso and Milburn, 1979; Steinbeck et al., 2007), though death might occur well after the larvae had experienced the entrainment process.

Most fishes produce large numbers of offspring that die naturally during early life (Dahlberg, 1979; Buckley, 1984), leaving uncertainty as to the ultimate effect of egg and larval entrainment on adult population numbers (Heimbuch et al., 2007). Natural, or background, sources of larvae and egg mortality are well known (e.g., described by Heath, 1992), and include endogenous as well as exogenous factors. Endogenous mortality is typically associated with genetic or developmental abnormalities, with documented endogenous mortality rates for fish eggs ranging as high as 90% (Heath, 1992). Exogenous factors include physical damage, pollution, disease, starvation and predation, and these are affected by prevailing environmental conditions. As most aquatic systems are characterized by high biotic and abiotic variability, environmental conditions experienced by larval fish are often inconsistent and highly patchy in space and time. Some assemblages of fish early life stages may experience favorable environmental conditions and be less likely to suffer immediate mortality through natural processes, while other groups may be at high risk of near term mortality. Depending on physical processes and habitat characteristics of a given water body, environmental conditions may change rapidly and a group of fish might experience both favorable and unfavorable conditions in a single day. Thus, within a single body of water the condition of individual fish and their likelihood of experiencing natural mortality can change rapidly across space and time. The wide range of sources and rates of natural mortality confounds the ability to evaluate the ultimate population-level effects of entrainment. However, determining the relative condition of entrained larvae prior to and as a result of entrainment has the potential to improve our understanding of ultimate population level consequences. That is, if entrained larvae are in very poor condition prior to entrainment and imminently likely to die in the short term regardless of entrainment, the effects on the population because of entrainment are likely relatively minor.

Larval fish experiencing prolonged periods of low prey availability and high energetic costs may be at risk of direct starvation mortality, but also rendered more vulnerable to other stressors (e.g., Skajaa et al., 2003) including entrainment. Conversely, entrained larval fishes subjected to thermal changes, turbulence, and other stressors related to the entrainment process may experience physical and metabolic alterations that render the fish less likely to survive upon return to the environment. Methods of identifying the condition of larvae and eggs subjected to entrainment are needed. Specifically, approaches are needed that could identify organisms unlikely to survive independent of entrainment (due to inevitable natural mortality), evaluate the change in condition of individual larval fish that survive the entrainment process, and relate the time over which these stressors act. In the United States, such studies are particularly timely as on August 15, 2014, the United States Environmental Protection Agency issued Final Regulations to Establish Requirements for Cooling Water Intake Structures at Existing Facilities and Amend Requirements at Phase I Facilities (Final Rule), establishing compliance requirements under Section 316(b) of the Clean Water Act (EPA, 2014). Historically, entrainment studies have assumed that all entrainable organisms were alive prior to entering cooling intake systems and died once entering the system (EPRI, 2011). However, this Final Rule mandates that large facilities quantify entrainment to support setting technology-based performance standards for minimizing environmental impacts that are driven by the facility's entrainment mortality levels.

Quantification of nucleic acid ratios (RNA:DNA) provides a short-term measure of condition and growth, and is based on the notion that DNA concentrations within individual cells remain fairly constant while RNA concentrations increase as protein synthesis increases (Buckley, 1980; Ferron and Leggett, 1994; Suthers et al., 1996). Thus, a recently well-fed, metabolically active, growing individual should have a relatively high RNA:DNA ratio compared to a starving, metabolically inactive individual (e.g., Richard et al., 1991). The measurement of nucleic acid ratios has been applied to assess individual condition of a variety of aquatic organisms, including phytoplankton (e.g., Berdalet and Dortch, 1991), zooplankton (e.g., Gorokhova and Kyle, 2002; Gorokhova, 2003), macroinvertebrates (e.g., Kyle et al., 2003), larval fish (e.g., Clemmesen, 1994; Pepin et al., 1999; Gwak et al., 2003) and juvenile/adult fish (e.g., Smith and Buckley, 2003). The approach has proved particularly appropriate for studies of larval fish, which display potentially rapid, nearly continuous growth of purely somatic tissues (i.e., energy available for larval fish growth is not diverted to molting or gonadal tissue). In addition, since whole larval fish can be readily analyzed for RNA:DNA ratios, potential biases associated with inconsistent tissue sampling are not a concern (Segner and Verreth, 1995; Selleslagh and Amara, 2013).

Several studies have documented that RNA:DNA ratios reflect short-term growth of larval fish in a laboratory setting (e.g., Clemmesen, 1994; McNamara et al., 1999) and have demonstrated variability in RNA:DNA ratios in situ across habitats (e.g., Robinson

and Ware, 1988; Clemmesen et al., 1997; Höök et al., 2008) or over time (e.g., Voss et al., 2006). There are various potential advantages of RNA:DNA ratios over other measures of organism condition. Most noteworthy, whereas many traditional measures of growth and condition integrate feeding history and energetic utilization over the whole life-time of an organism, nucleic acid ratios provide a measure of recent growth and condition (i.e., past 1–3 days; Buckley et al., 1999). Even for organisms such as fish that demonstrate approximately continuous growth, many traditional approaches for estimating growth rates involve repeated sampling and are susceptible to measurement biases (e.g., errors in aging fish using otoliths or scales; Bradford, 1991). Nucleic acid ratios however provide a point-in-time and potentially unbiased measure of individual condition and growth. Moreover, RNA:DNA ratios are strongly related to starvation across a variety of larval fish species (e.g., Meyer et al., 2012) and could potentially provide an index of imminent starvation mortality, a primary source of natural larval mortality.

Entrainment mortality has traditionally been calculated with the assumption that 100% of the mortality is caused by entrainment, despite the fact that natural sources of mortality exist in both marine and freshwater systems and that mortality levels can be very high (Dahlberg, 1979; Fuiman and Werner, 2002; EPA, 2006). Few studies at facilities have attempted to differentiate natural mortality numbers against facility (entrainment) related mortality (EPRI, 2011). Direct observations of individual organism conditions have been applied in few, highly localized studies, where researchers attempted to quantify facility mortality against background mortality. In this paper, we evaluate the potential utility of using RNA:DNA ratios as a tool in assessing the condition of early life stages of fishes subjected to entrainment by cooling water intake systems. We examine the methodological, ontogenetic and environmental sources of variability in RNA:DNA ratio values. Moreover, we analyze published literature through a meta-analysis and secondary analysis to consider, (a) the length of time larval fish can starve and still resume feeding and ultimately survive and (b) the responsiveness of RNA:DNA ratios to starvation (and potentially other stressors) in larval fish. We finally offer recommendations for how facility scientists might integrate the use of RNA:DNA ratios into their assessments of background mortality and the lethal and sublethal effects of entrainment on early life history stages of fishes.

## 2. Sources of RNA:DNA variation

While RNA:DNA ratios have been shown to qualitatively and potentially quantitatively reflect growth and condition of a broad array of larval fishes, there exist multiple sources of variation that should be recognized when generalizing results across taxa and environmental conditions. Primary sources of nucleic acid ratio variation include methodological, ontogenetic, and temperature effects.

### 2.1. Methodology

One advantage of evaluating RNA:DNA ratios is that it is possible to quantify nucleic acid ratios of a large number of larval fish in a short amount of time. Samples are often analyzed in a 96-well microplate and, even with replicates, researchers can analyze 20–40 individual samples (i.e., larval fish) in a single plate with the time from preparation of samples to analysis as low as 8 h. The ability to generate this much data in so short a time makes this a potentially cost-effective tool for the analysis of large numbers of individuals collected during entrainment sample events.

Researchers should develop and implement strict collection methodologies for organisms included in RNA:DNA evaluations, as these methods have the potential to strongly influence resulting values and confound interpretation. For example, the physical condition of larval fish upon collection has strong potential to affect RNA:DNA values. For many fishes, the larval stage is particularly fragile, and the process of entrainment and collection will subject specimens to various physical stresses (reviewed in Marcy et al., 1978; Mayhew et al., 2000; EPRI, 2009, 2014). RNA:DNA ratios of a larval fish's head are lower than those calculated for its whole body (Olivar et al., 2009), thus inclusion of incomplete specimens (e.g., missing head or part of body) may bias RNA:DNA measurements. Fish condition and RNA:DNA ratios are also affected by the collection process itself. Theilacker (1978) noted the decline in specimen condition of larval northern anchovy after only 10 min spent in a net. Chícaro (1997) similarly suggested that stress of being held in a net may have had a greater effect on mortality of larval herring than availability of food. Time of collection may also affect RNA:DNA calculations. Larval fishes may exhibit cyclical differences in RNA:DNA throughout the day not in response to changes in temperature but potentially in response to daily differences in feeding patterns or endocrine activity (Rooker and Holt, 1996; Ching et al., 2012). Researchers can potentially reduce some of these sources of variation by maintaining consistent sampling methods, e.g., holding specimens for a specified length of time in the net, processing samples as quickly as possible, and always sampling at the same time of day.

Consistent methodologies are also important in laboratory analyses. Currently, the general, wide-spread approach for quantification of RNA:DNA ratios involves fluorometrically measuring nucleic acid concentrations of samples before and after digestion with nucleases (RNase and/or DNase). Samples can be analyzed immediately following collection but are typically stored for a period of time before nucleic acid determination. RNA can degrade quite quickly and the prevalence of RNAse enzymes in the environment can complicate the process. To minimize nucleic acid degradation from sources of nucleases present in surroundings, samples are stored at cold temperatures (e.g., −80 °C) or storage reagents are employed, such as RNAlater® (a super-saturated salt solution which greatly decreases the rate of nucleic acid degradation; Gorokhova, 2005). Nucleic acid extraction is performed in a clean environment and tools used during the process are sprayed liberally with RNAse decontamination solutions. Researchers may extract nucleic acids from individual fish (e.g., Gronkjaer et al., 1997; Tanaka et al., 2008) or pool specimens until an appropriate amount of sample is achieved (e.g., Canino, 1994; McNamara et al., 1999). The exact method for nucleic acid extraction from tissues differs among researchers, but generally includes some combination of chemical extraction buffers, centrifugation, and physical homogenization. Similarly, exact methods for measuring nucleic acid concentrations differ, but generally involve treatment with a fluorescing agent (e.g., Ethidium Bromide; Ribogreen®) and quantification of fluorescence before and after treatment with some nuclease, to first quantify total nucleic acid concentration and then quantify RNA or DNA alone. Specifically, to estimate nucleic acid concentrations, sample fluorescence values are compared to fluorescence values of RNA and DNA standard curves of known concentration. The standards are treated similarly to the samples in terms of preparation and techniques designed to minimize nucleic acid degradation. As before, standards differ among protocols and a large variety of types of standards have been employed for this purpose. Correct estimation of both nucleic acids is important, as errors in either the numerator ([RNA]) or denominator ([DNA]) can strongly influence nucleic acid ratios (Hovenkamp and Witte, 1991; Suthers et al., 1996).

As previously described in this section, there is the potential for large differences among RNA:DNA quantification protocols related to particular storage and extraction methods, as well as the fluorescing agents, nucleases and standards employed (Bergeron, 1997). The potential implications of these methodological differences on resulting RNA:DNA values are not obvious. To address this knowledge gap, Caldarone et al. (2006) used four separate nucleic acid quantification protocols employed by five different labs to measure RNA:DNA ratios of replicate tissue samples. While the different protocols and research groups yielded qualitatively similar results (i.e., ranking of replicate sample RNA:DNA values was consistent within protocols), actual RNA:DNA values were quite variable among protocols (57.1% of RNA:DNA variation attributed to methodological protocol; Caldarone et al., 2006). To minimize RNA:DNA variation, Caldarone et al. (2006) proposed standardizing RNA:DNA values by a protocol specific slope ratio, being the ratio of (a) slope of a RNA standard concentration versus fluorescence curve to (b) slope of a DNA standard concentration versus fluorescence curve. Such standardization greatly reduced methodological effects, resulting in 3.4% of RNA:DNA variation attributed to methodological protocol (Caldarone et al., 2006). Subsequent studies have employed this technique to allow for comparison of samples analyzed in different ways (e.g., Meyer et al., 2012) and it should be adopted as a standard analytical approach.

### 2.2. Ontogenetic stage

Larval fish feeding varies with ontogeny, with newly hatched larvae deriving nutrition endogenously from a yolk sac while exogenously feeding larvae have access to an expanded prey base as swimming ability and gape size increases. Reliance on yolk sac stages may lead to less variation between individual RNA:DNA values (Gronkjaer et al., 1997; McNamara et al., 1999) because the ratios are less likely to reflect environmental conditions (e.g., feeding success). RNA:DNA values of newly hatched larvae may also be affected by differences in nucleic acid production conferred by an individual's parents (Høie et al., 1999; Heyer et al., 2001). Given these potential sources of variation or non-variation (as the case may be), several studies specifically exclude yolk sac larvae from analyses (e.g., Rooker and Holt, 1996; Suthers et al., 1996). Once larvae begin feeding exogenously, variation of RNA:DNA values may increase as a multitude of external factors may affect larval feeding, growth and condition (Gronkjaer et al., 1997; Catalán et al., 2006). Moreover, during early endogenous feeding larval fish develop quite rapidly and differential morphological, physiological and behavioral development may all contribute to high inter-individual variation of RNA:DNA ratios (Robinson and Ware, 1988; Bergeron, 1997; McNamara et al., 1999).

As larval fishes continue to grow and develop past the initial exogenous feeding stage, variability among individual RNA:DNA values within a population is expected to decrease and RNA:DNA values may become more strongly correlated with environmental conditions (e.g., Robinson and Ware, 1988; Cuhna et al., 2003; Catalán et al., 2006). Some researchers have suggested that when RNA:DNA values become less variable and begin to reflect environmental conditions, this is an indication that individuals who were unable to initiate feeding or otherwise under-performed have been removed from the population (e.g., Gronkjaer et al., 1997; McNamara et al., 1999; Pepin et al., 1999). Nucleic acid ratios of relatively small larval and metamorphosing fish are expected to react more rapidly to starvation (Rooker and Holt, 1996) and thus when small larvae are present in field samples, higher percentages of the overall catch may be classified as "starving" (Robinson and Ware, 1988). Response of RNA:DNA ratios to environmental factors also differs with size class (e.g., Clemmesen et al., 1997; Lee et al., 2006; Tanaka et al., 2008). It may therefore be most appropriate to compare nucleic acid ratios of fishes at similar developmental stages (e.g., Clemmesen et al., 1997; Gronkjaer et al., 1997; Kimura et al., 2000), and this has become standard practice in RNA:DNA starvation studies.

### 2.3. Temperature effects

Temperature has an overriding influence on physiological rates of larval fish and may both directly and indirectly affect RNA:DNA values. Metabolic activity (Bisbal and Bengtson, 1995; Rooker et al., 1997; Suneetha et al., 1999), growth (Buckley, 1984), and availability of prey (Clemmesen et al., 1997; Catalán et al., 2006) vary with temperature. Several studies have collected larval fish in the field and attempted to relate RNA:DNA values to environmental temperature (e.g., Suthers et al., 1996; Höök et al., 2008). However, it is not clear if such in situ relationships reflect a direct response to temperature or an indirect response as temperature may influence or be correlated with other important determinants of larval feeding and growth (e.g., prey availability, water clarity). Perhaps more important for RNA:DNA interpretation, temperature can directly affect the relationship between RNA:DNA and somatic growth rates of larval fish. That is, since metabolic processes tend to increase with temperature, a lower concentration of enzymes must be synthesized to catalyze reactions at a comparable rate at a warm temperature versus a cool temperature. Several studies aiming to relate RNA:DNA to fish growth have either developed temperature-specific relationships or explicitly included temperature in their predictive equations (e.g., Tanaka et al., 2008). For example, Brightman et al. (1997) found that, for larval red drum, RNA:DNA could predict protein growth very well at a given temperature; however, when considering the full suite of temperatures in their study (which ranged from 20 °C to 32 °C), the ability of RNA:DNA to accurately predict protein growth declined. The effect of temperature on the RNA:DNA–growth relationship is likely to vary with species and life-stage (e.g., Rooker et al., 1997; Voss et al., 2006), further complicating interpretation.

Larval fishes subject to entrainment may experience rapidly differing temperature regimes. Previous studies have demonstrated that quick and dramatic changes in temperature ($\Delta T$) can lead to mortality of a number of larval fishes (Cada et al., 1982; Bamber and Turnpenny, 2012), and $\Delta T$ in concert with other stressors such as residual chemicals from the chlorination process may elicit synergistic negative effects on larval fishes (Bamber and Turnpenny, 2012). From the perspective of using RNA:DNA values to assess the condition of entrained larval fish, $\Delta T$ may also directly influence RNA:DNA values and further confound interpretation of RNA:DNA ratios. That is, depending on whether (a) larval fish are collected prior to or after to experiencing $\Delta T$ and (b) the duration of elevated temperature exposure, measured RNA:DNA values may be lower than in the absence of such exposure. Such confounding effects may be mitigated by sampling in warmer systems or during warmer seasons, where differences between ambient water temperatures and water temperatures within the power plant are potentially less severe. Nonetheless, the potential influence of temperature is an important methodological consideration.

## 3. Ability of RNA:DNA to assess stress and likelihood of mortality

While RNA:DNA values are often used to assess relative growth rates of larval fish, when evaluating potential impacts of entrainment it may be more useful to assess whether entrained larval fishes are robust, and likely to survive despite entrainment, or in very poor condition and facing immediate risk of mortality from non-entrainment-related stresses. Several researchers have

**Table 1**
Studies identifying a critical RNA:DNA ratio below which an individual larval fish is classified as starving.

| Study | Species name and life stage | Field study location | Critical RNA:DNA ratio | Percentage of field-caught specimens below critical RNA:DNA ratio |
|---|---|---|---|---|
| Chícaro (1997) | *Sardina pilchardus* | Algarve's continental shelf, southern Portugal | 1.3 | 4.64% |
| Gronkjaer et al. (1997) | *Gadus morhua* | Bornholm Basin, Baltic Sea | 1.5 | Up to 30%, varies with location and life stage |
| Kimura et al. (2000) | *Sardinops melanostictus* | Along Kuroshio current, southern Japan | 1.17 temp. <17.5 °C<br>1.32 temp. >17.5 °C | 0% |
| Robinson and Ware (1988) | *Clupea harengus pallasi* | Strait of Georgia, western Canada | 2.06 | Up to 60% of early life stages, down to 0% latter life stages |
| Rooker et al. (1997) | *Sciaenops ocellatus* | Aransas Estuary, Gulf of Mexico | RNA:DNA = 1.171 + 0.330*SL (mm) | <5% |
| Suneetha et al. (1999) | *Clupea harengus* | Laboratory | 2.5 | N.A. |
| Tanaka et al. (2008) | *Thunnus orientalis* | Northwestern Pacific Ocean | <5 mm, 25 °C:<br>RNA:DNA = 3.73–0.29 * SL (mm)<br>>5 mm, 25 °C:<br>RNA:DNA = 2.83–0.14 * SL (mm)<br><5 mm, 28 °C:<br>RNA:DNA = 5.39–0.70 * SL (mm)<br>>5 mm, 28 °C:<br>RNA:DNA = 1.24–0.099 * SL (mm) | 4.3–25.8% depending on location, temperature and cohort examined |
| Westerman and Holt (1994) | *Sciaenops ocellatus* | Laboratory | 2.45 | N.A. |

attempted to assess the condition of larval fishes using RNA:DNA ratios empirically through a *critical ratio* approach, or the establishment of a ratio below which an individual is classified as starving and likely to experience imminent mortality (Table 1). Some researchers consider the critical ratio to be constant, though several studies have demonstrated that the critical RNA:DNA ratio varies with fish size and ambient temperature (e.g., Robinson and Ware, 1988; Rooker et al., 1997; Kimura et al., 2000; Tanaka et al., 2008). In general, critical RNA:DNA ratios for larval fishes seem to fall between 0.5 and 3. When using the critical ratio to assess the overall status of a fish population, the proportion of individuals with RNA:DNA values below the critical ratio is typically low (Table 1) but can vary with prey availability (e.g., Robinson and Ware, 1988; Lee et al., 2006; Tanaka et al., 2008) and temperature (Brightman et al., 1997; Kimura et al., 2000). RNA:DNA values may also be lower when fishes are quite young because under-performing (e.g., poor feeding) individuals, with low RNA:DNA values, are still contributing to sample estimates of condition (Chícaro et al., 1998). Researchers attempting to compare laboratory-derived critical ratios to field-caught larval populations should exercise caution, and consider and identify stresses imposed by ambient conditions (Canino et al., 1991; Westerman and Holt, 1994).

In addition to quantifying risk of starvation, researchers have related RNA:DNA ratios of fishes to other forms of stress, such as chemical exposure. Studies have examined a diversity of fish species and life stages, including larval fish. Not surprisingly, chemical concentrations which elicit a strong effect in RNA:DNA values vary with species, life-stage and the specific chemical evaluated. Nonetheless, RNA:DNA have been shown to decrease when fishes are exposed to various organic and inorganic chemicals, including benzophenone (sometimes used in sunscreen), ethyl acetate (used in a wide range of applications), methyl parathion (a pesticide), para-cresol (intermediate compound used in the production of many other chemicals), hexavalent chromium (byproduct of industrial processes), cadmium, mercury and mixture of chemicals (Kearns and Atchison, 1979; Barron and Aldeman, 1984; Parrott and Sprague, 1993; Miliou et al., 1998; Aditya et al., 2002; Li et al., 2014). From, the perspective of evaluating effects of entrainment it is therefore important to consider such effects of chemical exposure, especially if attempting to compare RNA:DNA values across systems.

Finally, researchers have also attempted to correlate RNA:DNA ratios with behavior-related stresses. Faria et al. (2011a) found that RNA:DNA ratios varied positively with critical swimming speed and suggested that better condition led to better swimming ability for *Spartus aurata* larvae. Faria et al. (2011b) also attempted to quantify the relationship of RNA:DNA to the critical swimming speed of Senegalese sole. While they did observe a trend of increased critical swimming speed at higher RNA:DNA values, this relationship was not statistically significant (Faria et al., 2011b). Similarly, Elliott and Leggett (1998) were unable to detect differences in RNA:DNA ratios of larval capelin exposed to a predator or not, indicating that RNA:DNA ratios may not be effective measures of behavior. It is likely that variation in RNA:DNA ratios of a population of fishes that can be attributed to other factors, such as temperature and starvation, would outweigh detected differences in behavior stressors in field-collected specimens, and Elliott and Leggett (1998) specifically noted that the lack of differences was likely due to the high variability in RNA:DNA ratios. Again, behavior is simply another source that may affect RNA:DNA ratios, albeit at a very small level of measurement.

## 4. Literature-based analyses of utility of RNA:DNA

One potentially valuable use of RNA:DNA ratios in entrainment evaluations involves assessing whether or not a fish was in poor condition (i.e., starving) and likely to die before being entrained. Here, we more broadly and systematically consider the ability of RNA:DNA ratios to reflect starvation (a major source of larval fish mortality) and the time being starved to reach the *point-of-no-return* (PNR). The PNR is the threshold at which 50% of unfed larvae are unable to resume feeding even if offered adequate prey (Blaxter and Hempel, 1963), an assumed threshold of highly certain mortality. We use PNR as a surrogate for understanding the relationship between RNA:DNA ratios and potential long-term negative impacts

on larval fishes due to entrainment stress. We conducted a meta-analysis from published research articles evaluating the response of larval fish RNA:DNA values to starvation and a secondary analysis of studies estimating the time to reach PNR.

### 4.1. Meta-analysis of starvation effects on RNA:DNA values

Our meta-analysis was designed to evaluate the response of larval fish RNA:DNA values to starvation. In order to be included in this analysis, a study had to (1) be conducted in a laboratory setting, i.e., under controlled settings; (2) include groups of starved and non-starved fishes being held in otherwise identical conditions; and (3) provide a mean RNA:DNA value plus variation (standard deviation or standard error) for both starved and non-starved fishes for at least two time periods post-onset of starvation. To identify studies to be included in the meta-analysis, we conducted a literature search using the Thomson Reuters Web of Science database (Thomson Reuters 2014) on May 10, 2014 using the search term "larval fish RNA: DNA starvation". This search returned 43 papers, 10 of which were included in our final analysis (76 individual records; Table 2). These 10 papers examined 9 different species, of which 6 were marine, 1 was freshwater and 2 were euryhaline.

We restricted our analyses to the larval life stage in order to avoid confounding factors related to methodological variation described in Section 2.1. For each study, we extracted mean and error values for each day that a starved and non-starved group of larval fishes, otherwise held in identical conditions, were compared. That is, for most papers we calculated greater than one effect size per paper. Specifically, we included more than one value per paper if more than one experiment was conducted and reported in terms of different temperature regimes (e.g., Suneetha et al., 1999; Tanaka et al., 2008), starvation began on different days of the experiment (e.g., Suneetha et al., 1999; Tanaka et al., 2008), or researchers performed replication of the entire experiment (e.g., McNamara et al., 1999). We extracted information from tables whenever possible but, when necessary, we measured mean and error from figures using ImageJ image analysis software (Rasband, 2009). When studies reported error as standard error, we used reported sample sizes to calculate standard deviation. If a study reported a range of individuals for a sample size (e.g., 8–12 individuals collected and analyzed for nucleic acid content on each day of the experiment), we assumed the highest possible sample size when calculating the pooled standard deviation. We used pooled standard deviation to calculate effect sizes (see below). We also noted experimental temperatures, mean length of larvae at hatch, mean length of larvae when RNA:DNA was quantified, duration of starvation and days since hatch when RNA:DNA was quantified.

A variety of effect sizes exist for evaluating responses of experimental versus control treatments. Rather than potentially bias our analysis by selecting a single response ratio, we calculated both Hedges $g$ (adjusted for small sample size; Hedges and Olkin, 1985) and log response ratio (Hedges et al., 1999). For Hedges $g$ we compared the difference between RNA:DNA values of fed and starved fishes, and for log response ratio we calculated the natural logarithm of the quotient of RNA:DNA values of fed fish over starved fish (i.e., for both effect sizes, a positive effect size indicates that RNA:DNA values of fed fish exceeded values of starved fish). As expected, these analyses indicated that starved larval fish do indeed express lower RNA:DNA values, as compared to feeding fish: log response ratio = 0.280 (0.263–0.296, 95% CI); $g$ = 0.428 (0.333–0.523, 95% CI). Effect sizes were quite variable among studies and appeared to be associated with study design and study organism characteristics. To evaluate this variability, we correlated both effect sizes (log response ratio and $g$) with size at hatching (length in mm; $n$ = 41), maximum experimental temperature ($n$ = 76) and duration of starvation ($n$ = 76; Table 2). While we did not find strong associations between effect sizes and size at hatching ($r$ = +0.04 and +0.06) or temperature ($r$ = +0.01 and −0.05), both effect sizes were significantly associated with duration of starvation ($r$ = +0.48 and +0.49; Fig. 1).

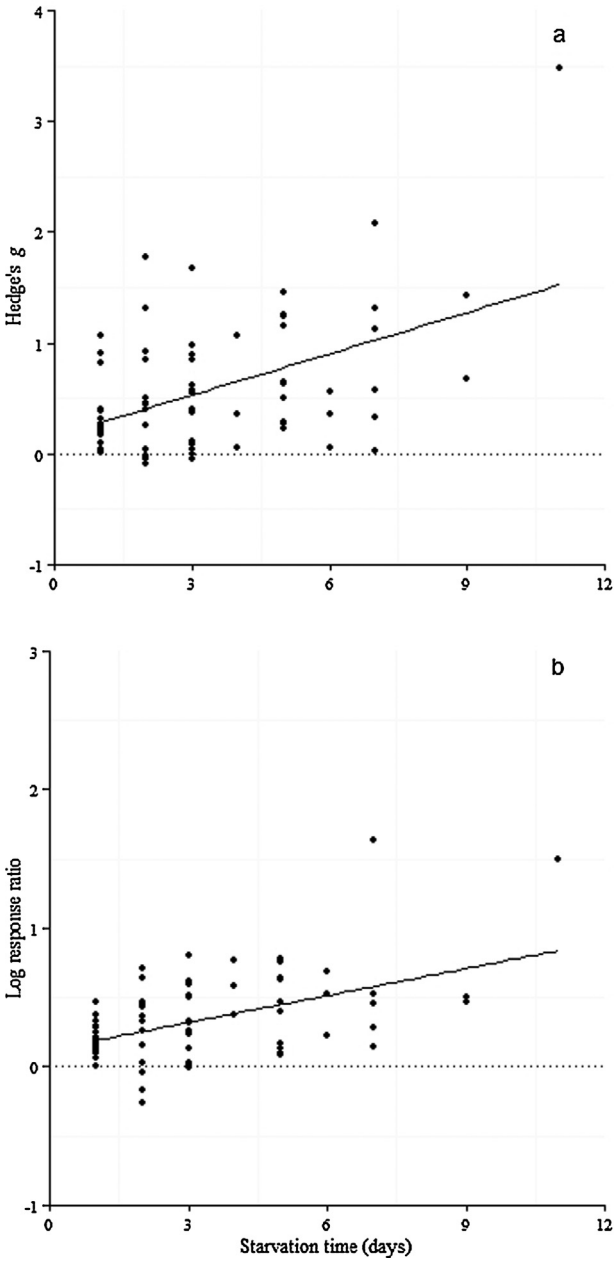


**Fig. 1.** Effect sizes from experiments evaluating larval fish RNA:DNA responses to starvation compared to duration of starvation: (a) Hedge's $g$ ($r$ = +0.49; $n$ = 76) and (b) log response ratio ($r$ = +0.48; $n$ = 76).

Meta-analyses have the potential to be influenced by publication bias, whereby not all studies are equally likely to be published and ultimately included in a meta-analysis (Møller and Jennions, 2001). In particular, studies demonstrating a strong effect between experimental and control groups may be more likely to be published. To evaluate the potential for publication bias, we plotted funnel plots (log effect size versus log sample size; raw data available in Table 2) for both effect sizes and calculated Rosenthal's failsafe number with $g$ as an effect size (Rosenthal, 1991; Møller and Jennions, 2001). The funnel plots (not presented) did not reveal evidence of systematic bias and Rosenthal's failsafe number was quite large, 2266. While these analyses cannot disprove the existence of

**Table 2**
Studies included in meta-analysis of starvation effects on RNA:DNA values. Dashes indicate data not reported.

| Citation | Species name | *n* fed | *n* starved | Mean size at hatch (mm) | Maximum water temperature (°C) | Duration of starvation (days) | Hedge's *g* | Log response ratio |
|---|---|---|---|---|---|---|---|---|
| Brightman et al. (1997) | *Sciaenops ocellatus* | 2 | 14 | 1.7 | 25 | 6 | 0.35 | 0.53 |
| Canino (1994) | *Theragra chalcogramma* | 20 | 20 | 4.42 | 12 | 1 | 0.18 | 0.11 |
| | *Theragra chalcogramma* | 20 | 20 | 4.42 | 12 | 2 | 0.26 | 0.15 |
| | *Theragra chalcogramma* | 20 | 20 | 4.42 | 12 | 3 | 0.57 | 0.33 |
| Faria et al. (2011b) | *Solea senegalensis* | 56 | 39 | 4.89 | 22 | 4 | 0.06 | 0.37 |
| | *Solea senegalensis* | 56 | 66 | 4.86 | 22 | 2 | −0.02 | −0.17 |
| Gronkjaer et al. (1997) | *Gadus morhua* | 12 | 12 | – | 8.2 | 2 | 0.46 | 0.71 |
| | *Gadus morhua* | 12 | 12 | – | 8.2 | 3 | 0.37 | 0.62 |
| | *Gadus morhua* | 12 | 12 | – | 8.2 | 5 | 0.5 | 0.78 |
| | *Gadus morhua* | 12 | 12 | – | 8.2 | 6 | 0.56 | 0.68 |
| | *Gadus morhua* | 12 | 12 | – | 8.2 | 7 | 2.07 | 1.64 |
| Kimura et al. (2000) | *Sardinops melanostictus* | 13 | 11 | 5.28 | 15.8 | 1 | 0.31 | 0.13 |
| | *Sardinops melanostictus* | 6 | 12 | 5.28 | 15.8 | 2 | 0.85 | 0.64 |
| | *Sardinops melanostictus* | 11 | 11 | 5.28 | 15.8 | 3 | 0.89 | 0.51 |
| | *Sardinops melanostictus* | 6 | 12 | 5.28 | 15.8 | 4 | 1.07 | 0.59 |
| | *Sardinops melanostictus* | 11 | 11 | 5.28 | 15.8 | 5 | 1.26 | 0.65 |
| Segner and Verreth (1995) | *Clarias gariepinus* | 2 | 2 | – | 27.5 | 1 | 0.28 | 0.2 |
| | *Clarias gariepinus* | 2 | 2 | – | 27.5 | 3 | 0.98 | 0.81 |
| | *Clarias gariepinus* | 2 | 2 | – | 27.5 | 5 | 1.24 | 0.76 |
| McNamara et al. (1999) | *Gadus morhua* | 3 | 3 | – | – | 1 | 0.23 | 0.06 |
| | *Gadus morhua* | 3 | 3 | – | – | 3 | −0.05 | −0.01 |
| | *Gadus morhua* | 3 | 3 | – | – | 5 | 0.29 | 0.09 |
| | *Gadus morhua* | 3 | 3 | – | – | 7 | 1.31 | 0.46 |
| | *Gadus morhua* | 3 | 3 | – | – | 9 | 0.67 | 0.5 |
| | *Gadus morhua* | 3 | 3 | – | – | 11 | 3.48 | 1.5 |
| | *Gadus morhua* | 3 | 3 | – | – | 3 | 0 | 0 |
| | *Gadus morhua* | 3 | 3 | – | – | 5 | 0.65 | 0.09 |
| | *Gadus morhua* | 3 | 3 | – | – | 7 | 0.57 | 0.15 |
| | *Gadus morhua* | 3 | 3 | – | – | 9 | 1.43 | 0.46 |
| Suneetha et al. (1999) | *Clupea harengus* | 15 | 15 | – | 5.3 | 1 | 0.25 | 0.13 |
| | *Clupea harengus* | 15 | 15 | – | 5.3 | 3 | 0.04 | 0.02 |
| | *Clupea harengus* | 15 | 15 | – | 5.3 | 5 | 0.27 | 0.14 |
| | *Clupea harengus* | 15 | 15 | – | 8.3 | 1 | 0.1 | 0.1 |
| | *Clupea harengus* | 15 | 15 | – | 8.3 | 3 | 0.12 | 0.13 |
| | *Clupea harengus* | 15 | 15 | – | 8.3 | 5 | 0.23 | 0.1 |
| | *Clupea harengus* | 15 | 15 | – | 8.3 | 7 | 0.32 | 0.28 |
| | *Clupea harengus* | 15 | 15 | – | 8.3 | 1 | 0.2 | 0.16 |
| | *Clupea harengus* | 15 | 15 | – | 8.3 | 3 | 0.84 | 0.25 |
| | *Clupea harengus* | 15 | 15 | – | 8.3 | 5 | 1.16 | 0.4 |
| | *Clupea harengus* | 15 | 15 | – | 8.3 | 7 | 1.13 | 0.52 |
| | *Clupea harengus* | 15 | 15 | – | 11.3 | 1 | 0.22 | 0.12 |
| | *Clupea harengus* | 15 | 15 | – | 11.3 | 3 | 0.41 | 0.24 |
| | *Clupea harengus* | 15 | 15 | – | 11.3 | 5 | 0.64 | 0.46 |
| | *Clupea harengus* | 15 | 15 | – | 11.3 | 1 | 0.21 | 0.06 |
| | *Clupea harengus* | 15 | 15 | – | 11.3 | 3 | 0.57 | 0.26 |
| | *Clupea harengus* | 15 | 15 | – | 11.3 | 5 | 1.45 | 0.63 |
| Suthers et al. (1996) | *Macquaria novernaculeata* | 12 | 12 | 4.3 | 19 | 1 | 0.04 | 0.16 |
| | *Macquaria novernaculeata* | 12 | 12 | 4.3 | 19 | 2 | −0.09 | −0.26 |
| | *Macquaria novernaculeata* | 12 | 12 | 4.3 | 19 | 3 | 0.08 | 0.32 |
| | *Macquaria novernaculeata* | 12 | 12 | 4.3 | 19 | 4 | 0.36 | 0.77 |
| | *Macquaria novernaculeata* | 12 | 12 | 4.3 | 19 | 5 | 0.26 | 0.17 |
| | *Macquaria novernaculeata* | 12 | 12 | 4.3 | 19 | 6 | 0.05 | 0.22 |
| | *Macquaria novernaculeata* | 12 | 12 | 4.3 | 19 | 7 | 0.03 | 0.15 |
| Tanaka et al. (2008) | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 1 | 0.39 | 0.29 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 2 | −0.05 | −0.04 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 3 | 0.61 | 0.5 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 1 | 0.02 | 0.01 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 2 | 0.04 | 0.02 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 3 | 0.55 | 0.25 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 1 | 0.4 | 0.25 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 2 | 0.5 | 0.33 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 3 | 1.67 | 0.59 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 1 | 0.25 | 0.17 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 2 | 0.93 | 0.26 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 1 | 1.07 | 0.37 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 2 | 1.77 | 0.64 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 1 | 0.9 | 0.33 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 25 | 2 | 1.31 | 0.45 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 28 | 1 | 0.09 | 0.1 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 28 | 2 | 0.44 | 0.43 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 28 | 1 | 0.17 | 0.18 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 28 | 2 | 0.5 | 0.46 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 28 | 1 | 0.27 | 0.29 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 28 | 2 | 0.4 | 0.36 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 28 | 1 | 0.32 | 0.22 |
| | *Thunnus orientalis* | 12 | 12 | 3.2 | 28 | 1 | 0.82 | 0.46 |

**Table 3**
Primary studies included in the secondary point-of-no-return analysis.

| Citation | Species name | Family | Order | Size at hatch (mm) | Water temperature (°C) | PNR |
|---|---|---|---|---|---|---|
| Bisbal and Bengtson (1995) | *Paralichthys dentatus* | Paralichthyidae | Pleuronectiformes | 3.84 | 12.5 | 11.5 |
| | *Paralichthys dentatus* | Paralichthyidae | Pleuronectiformes | 3.84 | 21 | 6.5 |
| Chen and Fang (2007)[a] | *Tanichthys albonubes* | Cyprinidae | Cypriniformes | 2.7 | 26.2 | 8.5 |
| Ching et al. (2012) | *Epinephelus fuscoguttatus* | Serranidae | Perciformes | 2.8 | 27 | 2.8 |
| Dabrowski et al. (1986) | *Coregonus lavaretus* | Salmonidae | Salmoniformes | 9.9 | 16.2 | 11.1 |
| | *Coregonus peled* | Salmonidae | Salmoniformes | 10.5 | 16.2 | 13.2 |
| Dou et al. (2005) | *Paralichthys olivaceus* | Paralichthyidae | Pleuronectiformes | 2.64 | 15 | 7.7 |
| | *Paralichthys olivaceus* | Paralichthyidae | Pleuronectiformes | 2.58 | 18 | 5.2 |
| | *Paralichthys olivaceus* | Paralichthyidae | Pleuronectiformes | 2.62 | 21 | 4.2 |
| Gisbert et al. (2004) | *Paralichthys californicus* | Paralichthyidae | Pleuronectiformes | 2.7 | 18 | 7 |
| Huang et al. (2012) | *Cichlasoma managuense* | Cichlidae | Perciformes | 5.58 | 28 | 9.5 |
| Huang et al. (2007)[a] | *Acipenser schrenckii* | Acipenseridae | Acipenseriformes | – | 28 | 16 |
| Jonas and Wahl (1998) | *Sander vitreus* | Percidae | Perciformes | 8 | 20 | 8 |
| Kimura et al. (2000) | *Sardinops melanostictus* | Clupeidae | Clupeiformes | 5.28 | 15.8 | 6.3 |
| Margulies (1993) | *Euthynnus lineatus* | Scombridae | Perciformes | – | 27.1 | 1.5 |
| Overton et al. (2010) | *Gadus morhua* | Gadidae | Gadiformes | 4.4 | 10.2 | 8.5 |
| Primavera-Tirol et al. (2014) | *Epinephelus coioides* | Serranidae | Perciformes | – | 29.5 | 4 |
| Rice et al. (1987) | *Coregonus hoyi* | Salmonidae | Salmoniformes | 10 | 13.5 | Not observed |
| Shan et al. (2009) | *Miichthys miiuy* | Sciaenidae | Perciformes | 1.9 | 24 | 6 |
| Shan et al. (2008)[a] | *Oplegnathus fasciatus* | Oplegnathidae | Perciformes | 2.42 | 22 | 5 |
| Sirol et al. (1998) | *Hoplias* cf. *lacerdae* | Erythrinidae | Characiformes | 7 | 26.8 | Not observed |
| Torao (2012) | *Hypomesus nipponensis* | Osmeridae | Osmeriformes | 4.92 | 13 | 5 |
| Xiong et al. (2006) | *Anchorythroculter nigrocauda* | Cyprinidae | Cypriniformes | 4.04 | 28 | 11 |
| | *Parabotia fasciata* | Cobitidae | Cypriniformes | – | 30 | 5 |
| | *Parabotia fasciata* | Cobitidae | Cypriniformes | – | 28 | 5.5 |
| | *Parabotia fasciata* | Cobitidae | Cypriniformes | – | 26 | 7 |
| | *Parabotia fasciata* | Cobitidae | Cypriniformes | – | 24 | 8 |
| | *Parabotia fasciata* | Cobitidae | Cypriniformes | – | 22 | 9 |
| | *Parabotia fasciata* | Cobitidae | Cypriniformes | – | 20 | 8.5 |
| Yin and Blaxter (1986) | *Gadus morhua* | Gadidae | Gadiformes | 4.5 | 6.9 | 11 |
| | *Platichthys flesus* | Pleuronectidae | Pleuronectiformes | 2.6 | 9.5 | 10 |

[a] Additional PNR and temperature data taken from tables in these studies. See Section 4.2 for details.

publication biases, they indicate that our general effect size is likely not primarily a reflection of such biases, i.e., starvation does indeed lead to lower RNA:DNA values for larval fish.

### 4.2. Secondary analysis of time to reach point-of-no-return

Several researchers have suggested that PNR is inversely related to temperature (e.g., Bisbal and Bengtson, 1995), positively related to size at hatch (e.g., Rice et al., 1987) and responsive to some other species specific characteristics. Given that larval fish RNA:DNA ratios vary with temperature and size of an organism, we conducted a secondary analysis of primary literature to examine the effect of water temperature and size at hatch on time to PNR, and compared results of this analysis to those of the previously described meta-analysis. For the secondary PNR analysis, we conducted a literature search using the Thomson Reuters Web of Science database (Thomson Reuters 2014) on May 11, 2014 using the search term "larval fish point of no return". This search returned 48 papers, 20 of which were included in our final analysis (Table 3). The 31 individual records represented 10 marine species, 7 freshwater species, and 5 euryhaline species. We included studies that (1) were conducted in a laboratory setting; (2) defined the PNR as the point at which 50% of unfed larvae are unable to resume feeding even if offered adequate prey; and (3) provided an estimate of the number of days for starving fishes to reach PNR (Table 3). Three of the papers contained tables that listed additional temperature and PNR data for species and studies not already included in our analysis. Including this information added an additional 46 records to our water temperature analysis. We used correlations to assess the relationship between: (1) water temperature and PNR including only results from the studies found in our search, (2) water temperature and PNR including results from our search and the additional tabular data, and (3) size at hatch (mm) and PNR for studies found in our search.

The results of our PNR analysis (Fig. 2) are consistent with previous work, in that the time to reach the PNR appears to be negatively associated with temperature (original dataset, $n = 29$, $r = -0.29$, $p = 0.13$; expanded dataset, $n = 74$, $r = -0.20$, $p = 0.083$) and positively associated with size at hatch ($n = 20$, $r = +0.57$, $p = 0.009$). We further examined the association with water temperature and time to PNR, grouping by taxonomic order rather than considering individual species. The relationship held true for most orders, though Cypriniformes and Salmoniformes are an exception and displayed no relationship between water temperature and time to PNR (C. Foley, unpublished data). We similarly attempted to examine the relationship between size at hatch and PNR by order, but sample sizes were too small to provide meaningful comparisons. Two studies (Rice et al., 1987; Sirol et al., 1998) found no PNR for the species in question. We excluded these values from statistical analyses; however, it is noteworthy that these two studies targeted relatively large fishes (Fig. 2b).

## 5. Practical utility of RNA:DNA for evaluating entrainment effects

Our meta-analysis demonstrates that RNA:DNA ratios are clearly responsive to environmental conditions experienced by larval fish and reflective of starvation-induced stress and potential mortality. This indicates that RNA:DNA ratios may be may be useful for assessing the health of fish larvae subjected to entrainment. However, the secondary analysis indicates that the time it takes for a major form of stress acting on a larval fish that can be measured using RNA:DNA values (i.e., starvation) to lead to irreversible consequences is typically on the order of >5 days. This timeline varies

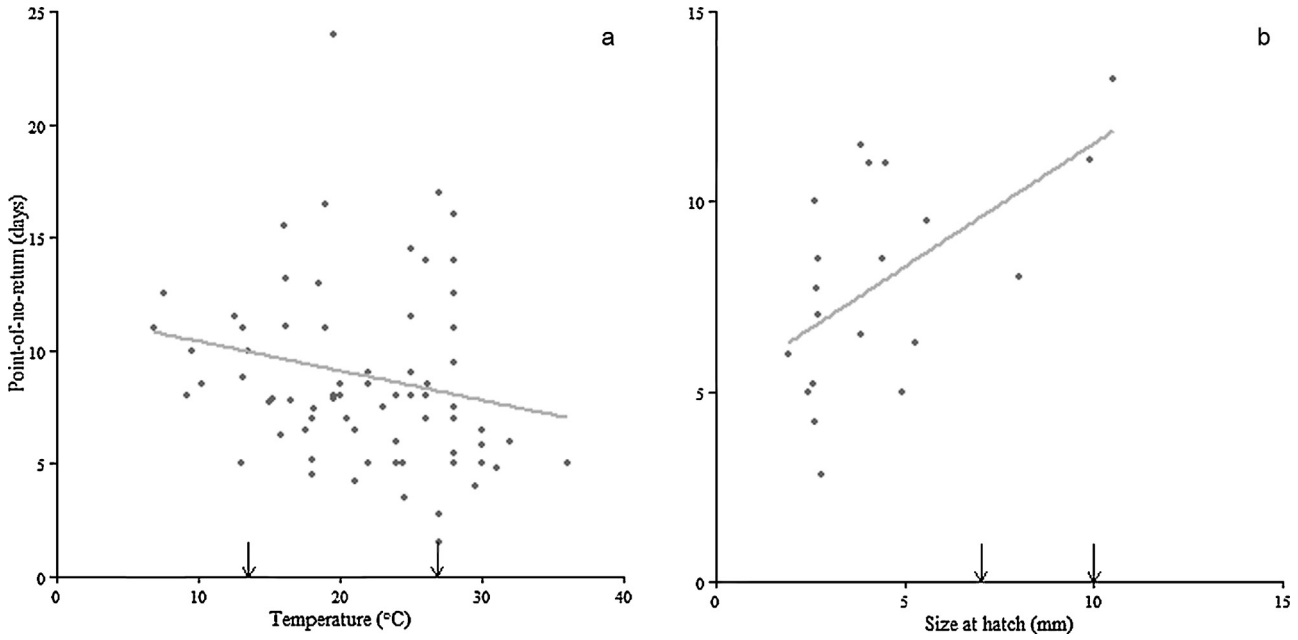


**Fig. 2.** Relationships between (a) temperature and point-of-no-return for all fishes analyzed, and (b) size at hatch and point-of-no-return. Data points reflect all studies listed in Table 3 plus additional data taken from tables within those studies. Arrows depict values from two studies where no PNR was determined.

with external factors (in this case, temperature and size at hatch), and it is likely that any entrainment-related stress affecting larval fish would similarly vary with external factors.

While our results reinforce the notion that interpretation of RNA:DNA ratios should be highly context dependent, they do not necessarily indicate that RNA:DNA ratios would not be a useful tool in assessing the effects of entrainment on larval fish. We caution that there are a multitude of methodological, taxonomic, ontogenetic and environmental sources of variation which should be accounted for when considering RNA:DNA patterns. In most cases, we suggest that complete accounting for these multiple sources of variation seems unlikely. Nonetheless, with consistent methodology and if the effects of ontogeny and temperature can be accounted for, e.g., by analyzing larval fish of a relatively uniform size and including a temperature adjustment term, then intra-specific, qualitative comparisons of RNA:DNA measurements may be insightful. For example, if a methodological- and taxa-specific critical ratio can be defined as a function of temperature and ontogenetic stage, then the proportion of entrained larval fish above or below this critical ratio could be tracked. Similarly, by tracking RNA:DNA values over time, one could assess whether there are trends in the condition of entrained fish, i.e., higher condition fish during one part of the year versus lower condition fish during another season. Such information could be used to understand background levels of mortality and population level impacts, and/or inform facility operational or technology options for reducing entrainment mortality and benefits valuation of early life stage impacts on fishes. In general, RNA:DNA values alone may be most informative when comparing multiple measurements taken in similar situations (e.g., temporal measurements from the same power plant, measurements on the same species at the same life stage from different spatial locations).

Researchers could potentially use RNA:DNA to assess direct effects of entrainment on larval fish condition, assuming minimal short-term immediate mortality. However, for multiple reasons, we suggest that the utility of this approach is likely to be limited at this time. First, the duration of entrainment from when a larval fish is first drawn in to a cooling system to when it is expelled back into the environment varies widely, based on the facility design. While RNA:DNA values are potentially responsive to short-term stresses, the time a fish is subjected to entrainment may be too short to detect meaningful changes in once-through cooling water facilities with short transport distances. Our meta- and secondary analyses demonstrate that the response of RNA:DNA values to starvation increases with duration of starvation, from 1 to 11 days, and the time to irreversible damage that can be measured using RNA:DNA is on the order of >5 days. Similar stresses on the order of <5 min seem unlikely to demonstrate a strong response. Second, the process of entrainment may expose larval fish to rapid, dramatic changes in temperature. While some studies have accounted for the influence of different fixed temperatures on RNA:DNA values (e.g., Tanaka et al., 2008), to our knowledge it is less clear how to systematically account for effects of rapid, temperature variation on nucleic acid ratios once the organisms are exposed to heat within the facility. To this point, we suggest that future studies could evaluate such influences.

Rather than looking at direct impacts related to entrainment, our review suggests that RNA:DNA ratios may be more useful in evaluating the condition of larval fish just prior to entrainment or at the time of entrainment and prior to heat exposure within the facility. This approach may be particularly useful if one aims to assess the relative composition (in terms of condition) of local populations that may be subject to entrainment or the relative condition of entrained larvae, provided such individuals, or subsamples, are processed shortly after entrainment and before being exposed to the full suite of potential entrainment stresses described previously. For example, if a large proportion of a local population of fishes demonstrates RNA:DNA values below a methodological-, temperature-, ontogenetic- and taxa-specific critical ratio, then the population may contain a large number of individuals likely to die from natural processes unrelated to entrainment. Similarly, researchers might compare variation in RNA:DNA values of larval fishes collected near a power plant intake or far from the intake versus variation in a group of larval fishes discharged from the facility. If the mean RNA:DNA ratio of the sample increases in thermal discharge samples from the facility, the values may be a sign that the weakest individuals are being removed from the population (i.e., those that might be likely to die anyway). If

the opposite trend is seen, entrainment stress may be negatively impacting the more healthy individuals of the population.

While we have focused on exogenously feeding larval fishes in the current study, RNA:DNA could also be used to assess other early life stages of fishes, including eggs and yolk sac larvae. These earlier stages often display lower variation in RNA:DNA values, which may render statistical analyses of resulting data more straightforward and allow for detection of significant effects with lower sample sizes. As these ontogenetic stages are unaffected by starvation, egg and yolk sac larvae RNA:DNA values cannot be related to this major source of mortality (similar to Peck et al., 2012). Instead, RNA:DNA values of these early life stages may be responsive to stresses that render them metabolically inactive. If methodological-, temperature-, and taxa-specific relationships between RNA:DNA and quality of yolk sac larvae or eggs can be developed, then the quality of individuals just prior to entrainment could be assessed. Moreover, if RNA:DNA of eggs and larvae respond rapidly to entrainment stresses, then the effects of the entrainment processes could be evaluated by comparing RNA:DNA of surviving individuals before and after entrainment.

It is worth noting that our review and analyses predominantly consider marine fishes. The majority of RNA:DNA studies on larval fish have taken place in marine systems, and our analyses reflect this. While cooling water intake systems are found in both freshwater, estuarine and marine systems, the majority of these systems (>90%) are found in freshwater environments (EPA, 2014). In order to maximize the utility of RNA:DNA as a tool in assessing entrainment stress, more studies examining RNA:DNA ratios in freshwater systems are needed.

## 6. Conclusions and recommendations

While RNA:DNA values clearly respond to starvation and provide an index of condition in a controlled laboratory environment (as demonstrated by our meta-analysis), quantitative interpretation of RNA:DNA values from in situ collected young fishes is not straightforward. RNA:DNA ratios will vary taxonomically and ontogenetically, and values may not be directly comparable among studies employing different methodologies. Moreover, environmental conditions, particularly temperature, influence how RNA:DNA ratios relate to somatic growth and risk of mortality. Given these multitude of influences, we conclude that the suitability of widespread adoption of RNA:DNA ratios to assess entrainment effects is limited at this time, especially within a regulatory framework. In particular, additional research is needed to understand to applications and limits of the index to organisms in freshwater environments. It is possible that researchers may wish to use RNA:DNA ratios to supplement other methods of evaluating the effects of entrainment on larval fish, and it is also possible that researchers might adopt use of RNA:DNA ratios to assess effects at a local scale. Researchers who wish to use RNA:DNA to assess the impact of entrainment stress on fish populations should follow a simple set of rules when designing their experimental studies.

1. When making comparisons across time and/or space, consider fishes at the same developmental stage or explicitly account for developmental stage in comparisons.
2. Collect information about the temperature in the area so as to assess and account for potential thermal effects on RNA:DNA values.
3. Follow consistent sampling and RNA:DNA quantification protocols, collecting specimens at the same time of day and treating them in the same manner from sample to sample and tray to tray (consider using the correction factor developed by Caldarone et al. (2006) to compare among trays and across studies).

RNA:DNA analyses may be most useful for assessing the condition of fish larvae susceptible to entrainment, evaluating whether fish larvae are likely to die from natural processes independent of entrainment, or assessing the relative condition of fish larvae which are still alive immediately following entrainment. We suggest that the former approach is currently more tractable and may provide a relative description of the likelihood of background mortality of entrainable organisms. However, to maximize the utility of the RNA:DNA technique, researchers might compare the mean and variation of RNA:DNA values of larvae among groups collected in an ecologically similar area unaffected by the intake system as well as at the intake and at the outfall of a cooling water intake facility. In addition, RNA:DNA results may be used as a complementary method of investigating entrainment condition, or stresses on larval fish populations, alongside analyses such as observation approaches that attempt to differentiated natural sources of mortality against facility-based mortality.

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